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Original Article

Exogenous colonization of carbapenem-resistant *Klebsiella pneumoniae* and vancomycin-resistant *Enterococcus* in preterm infants: a PFGE-based molecular epidemiology study

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Abstract

Introduction: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) and vancomycin-resistant *Enterococcus* (VRE) pose significant threats in neonatal intensive care units (NICUs) due to their high transmission potential and limited treatment options. Identifying the source of colonization and detection of predominant antibiotic-resistant genes are crucial for effective infection control measures.

Methodology: In this study, we analyzed 20 carbapenem-susceptible *Klebsiella pneumoniae* (CSKP) and 20 Vancomycin-susceptible *Enterococcus* (VSE) of meconium versus 20 CRKP and 20 VRE of rectal swabs isolates, respectively, from 20 preterm infants hospitalized in the NICU. Pulsed-field gel electrophoresis (PFGE) and arbitrarily primed PCR (AP-PCR) were used for molecular epidemiological analysis. The presence of carbapenemase (bla_{OXA-48}, bla_{NDM}, bla_{KPC}) and Vancomycin-resistance (*vanA, vanB, vanC*) genes was investigated by multiplex PCR.

Results: No predominant outbreak strain was detected, and isolates exhibited high genetic diversity, indicating an exogenous source of colonization of both CRKP and VRE. PFGE analysis revealed 24 distinct genotypes among CRKP and 25 among VRE isolates, with a clustering rate of 57.5%. The most commonly detected resistance gene in CRKP isolates was blaOXA-48 (50%), followed by blaNDM (35%) and blaKPC (10%). Among VRE isolates, only the vanA gene was present (85%).

Conclusions: The absence of a clonal outbreak and the detection of resistance genes primarily on plasmids indicate healthcare-associated transmission rather than endogenous selection. This highlights the critical role of hand hygiene and strict infection control measures in preventing multidrug-resistant pathogen colonization in vulnerable preterm infants.

Key words: Carbapenem-resistant *Klebsiella pneumoniae*; vancomycin-resistant *Enterococcus*; pulsed-field gel electrophoresis; nosocomial transmission; AP-PCR.

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Introduction

Members of the *Enterobacteriaceae*, particularly *Klebsiella pneumoniae* (*K. pneumoniae*), are important causes of community- and hospital-acquired soft tissue, bloodstream, and urinary tract infections [1,2]. Unnecessary and misuse of antibiotics makes *K. pneumoniae* resistant to most of them and become multidrug-resistant (MDR) strains. MDR-*K. pneumoniae* strains cause serious and life-threatening infections, especially in intensive care units (ICU). The spread of carbapenemases such as bla_{KPC}, bla_{IMP}, bla_{OXA-48}, and bla_{NDM} among *K. pneumoniae* through gene transfer causes MDR *K. pneumoniae* to acquire carbapenem resistance (CRKP) and limits the treatment options of these infections [1]. According to CHINET's report, while resistance to both meropenem and imipenem was 9.2% in 2010, it increased to 22.5% and 23.6%, respectively, for *Klebsiella* in 2023 [3].

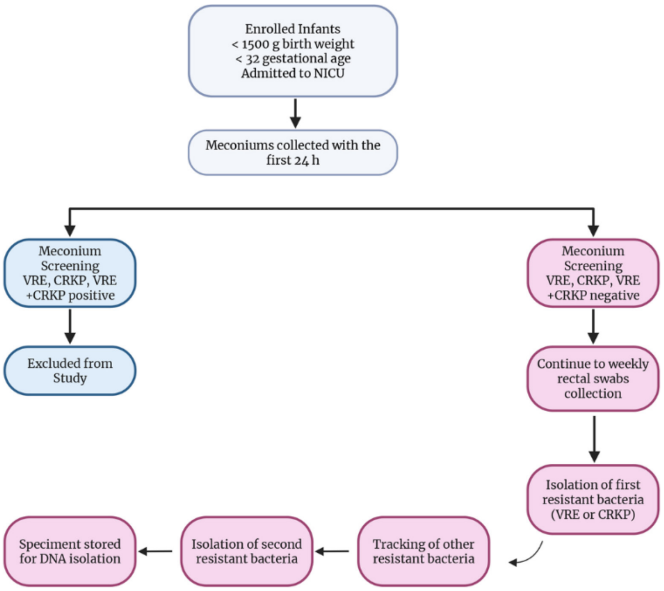
Because *K. pneumoniae* is a component of the human gut microbiota, every healthy individual has the potential to become a reservoir of MDR and CRKP due to unnecessary and misuse of antibiotics [4]. The patient room surfaces contaminated with MDR and CRKP strains can easily spread through the hands of healthcare workers and the gloves they touch to other patients [5]. Therefore, hand hygiene of health care workers and early detection of MDR and CRKP colonized in the intestine of hospitalized patients are playing an important role in the control of CRKP strains [6,7].

Infections caused by vancomycin-resistant *Enterococcus* (VRE) often involve intra-abdominal sites, the urinary tract, the bloodstream, surgical sites, vascular catheter sites, patients with hematological disorders, and prolonged hospitalization at the ICU [7,8]. VRE is a nosocomial pathogen and represents a

significant global health concern [9]. Due to limited antibiotic options, invasive infections caused by VRE are an important reason for mortality, and thus, active surveillance is underway to prevent VRE expansion [10]. *Enterococcus faecalis* (*E. faecalis*) is the most commonly isolated species, responsible for approximately 70% of human infections. Currently, VRE accounts for over 30% of enterococcal infections, with over 90% of VRE isolates identified as *E. faecium*. Conversely, only 5% to 10% of *E. faecalis* isolates show vancomycin resistance [11].

The high genomic plasticity of *E. faecium*, its ability to easily acquire new adaptive traits, and its higher rate of vancomycin resistance give the bacterium an advantage over *E. faecalis*. Furthermore, *E. faecium* may possess virulence and colonization factors, including hyaluronidase and enterococcal surface protein. These proteins may play a role in the adherence of the microorganism to host tissues and in the formation of biofilms [12-14]. CRKP and VRE can colonize in the intestine of patients endogenously as a result of different antibiotics that make commensals, such as *Enterococcus* and *K. pneumoniae,* develop resistance to selected antibiotics or exogenously through the transfer of both pathogens from unhygienic health care workers' hands to patients. The present study aimed to determine the sources of intestinal colonization with CRKP and VRE in preterm infants hospitalized in the intensive care unit.

Figure 1. Study design and workflow of resistant bacterial colonization monitoring in preterm infants admitted to the NICU.



Methodology

Study Design, Bacterial Isolates, and Identification of CRKP and VRE

Preterm infants with a birth weight of < 1500 g and a gestational age of < 32 weeks who were admitted to the neonatal ICU (NICU) and infants in whom resistant bacteria (VRE or CRKP) were not detected in meconium were included in the study. Meconium samples were collected within the first 24 hours after birth, and rectal swabs were obtained weekly thereafter. Upon detection of the first resistant organism (VRE or CRKP), samples were stored for further monitoring, isolation of a second resistant strain, monitoring for other resistant bacteria, and DNA extraction (Figure 1).

Sample collection was conducted between January

Table 1. Birth information and VRE/CRKP isolation dates of infants included in the study.

Baby no	Birth date (DD/MM/YYYY)	First date of isolation	Date of isoaltion of both VRE and CRKP
1	18.11.2021	06.12.2021--CRKP	13.12.2021
2	18.11.2021	30.11.2021--CRKP	13.12.2021
3	20.12.2021	07.02.2022--VRE	28.02.2022
4	01.12.2021	02.02.2022--CRKP	08.03.2022
5	24.02.2022	14.03.2022--CRKP	22.03.2022
6	23.03.2022	29.03.2022--CRKP	08.04.2022
7	13.04.2022	26.04.2022--VRE	05.05.2022
8	02.09.2022	26.09.2022--VRE	11.10.2022
9	02.12.2022	20.12.2022--CRKP	03.01.2023
10	16.12.2022	09.01.2023--VRE	17.01.2023
11	16.12.2022	17.01.2023--CRKP	30.01.2023
12	09.01.2023	13.02.2023--VRE	06.03.2023
13	07.01.2023	24.01.2023--CRKP	13.02.2023
14	01.06.2022	15.08.2022--VRE	01.08.2022
15	01.06.2022	28.06.2022--VRE	01.08.2022
16	21.06.2023	03.07.2023--VRE	24.07.2023
17	02.03.2023	24.04.2023--VRE	01.05.2023
18	02.03.2023	10.04.2023--VRE	10.05.2023
19	17.04.2023	16.05.2023--CRKP + VRE	16.05.2023
20	17.04.2023	01.05.2023--CRKP	16.05.2023

CRKP: Carbapenem-resistant Klebsiella pneumoniae; VRE: Vancomycin-resistant Enterococcus. Dates are given as day.month.year. "First date of isolation" refers to the initial detection of either CRKP or VRE in weekly rectal swab cultures. "Date of isolation of both VRE and CRKP" refers to the earliest date when both pathogens were detected in the same infant during hospitalization. Joined rows indicate twin infants.

2022 and August 2023 at the NICU of Istanbul Faculty of Medicine Hospital within the scope of our previously published study, the doctoral project of Dr. Ahmet AKTAŞ (Aktaş *et al.* 2024) (Table 1).

Rectal samples were collected weekly from preterm infants (n = 20) hospitalized in the NICU of Istanbul Faculty of Medicine Hospital. Conventional methods were used for the isolation of commensals *K. pneumoniae* by culturing meconium samples on Mac Conkey Agar (Oxoid, USA) and on Bile Esculin Agar (Biolab, Hungary) for isolation of *Enterococcus*. Whereas rectal swabs per week from the same patients were taken and cultured on Mac Conkey Agar with meropenem (1 mg/L) for CRKP and Bile Esculin Agar with vancomycin (6 mg/L) for isolation of VRE [15]. The Vitek2 (bioMérieux, France) automated system was used when needed.

K. pneumoniae isolates were classified as CRKP when they were resistant to at least one of the carbapenems (ertapenem, imipenem, or meropenem). *Enterococcus* spp. were defined as VRE when they were resistant to glycopeptides (both vancomycin and teicoplanin). The Kirby-Bauer disk diffusion method was used to evaluate susceptibilities to antibiotics [17]. The Vitek2 (bioMérieux, France) automated system was used only for confirmation of suspicious VRE or KRKP strains when needed.

Identification of Carbapenem Resistance Genes and Vancomycin Resistance Genes

Genomic DNA extraction was conducted using the QIAamp DNA Midi Kit (Qiagen, Hilden, Germany) on the QIAsymphony automated DNA extraction system (Qiagen, Hilden, Germany). The extracted DNA was stored at −80 °C until further analysis. The carbapenemase genes in CRKP isolates, including bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{OXA-48}, were detected using a multiplex polymerase chain reaction (PCR) protocol described by Poirel *et al.* [18]. Similarly, vancomycin resistance genes, including *vanA, vanB, and vanC*, were analyzed following the protocol previously described by Dutka-Malen *et al.* [19]. Amplification of the DNA was performed using the GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Waltham, MA, USA). PCR products were separated by electrophoresis on 1.5% agarose gels at 100 V for 1.5 h, stained with ethidium bromide, and visualized under UV light using a Kodak Gel Logic 200 imaging system

Molecular Epidemiological Analysis

AP-PCR

Clonal relationships among isolates were assessed using arbitrary primer sequence-based PCR (AP-PCR). The procedure was carried out following the protocol of Kurt *et al*. [20] with slight modifications. The reaction mixture (50 µL) consisted of 100 ng of genomic DNA, 100 pmol of the M13 primer (5′-GAGGGTGGCGGTTCT-3′), 0.5 units of Taq DNA polymerase (Vivantis Technologies, Malaysia), 200 µmol of a deoxynucleoside triphosphate mix, and a 10X amplification buffer. Banding patterns were analyzed using the GelCompar II software (version 6.6; Applied Maths, Sint-Martens-Latem, Belgium). Similarity of the banding profiles was calculated using the Dice correlation coefficient, and clustering was performed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) approach.

Pulsed-field gel electrophoresis (PFGE)

Isolates were stored at −80 °C until genotyping. Pulsed-field gel electrophoresis (PFGE) was performed as previously described [21]. Agarose plugs containing the isolates were digested with 20 units of the Xba-I restriction enzyme for *K. pneumoniae* isolates and 30 units of the Sma-I restriction enzyme for *Enterococcus* isolates. Gels were imaged using the Kodak Gel Logic 200 Imaging System (Eastman Kodak Company, Rochester, NY, USA). Banding profiles were analyzed using the GelCompar II software (version 6.6; Applied Maths, Sint-Martens-Latem, Belgium). Band pattern similarity was determined using the Dice coefficient, and clustering was conducted with the UPGMA method (optimization: 1.0, tolerance: 1.0, cutoff 85%). Isolates with band pattern similarities greater than 85% were classified as belonging to the same clone [22].

Visualization of Temporal Overlap

A Gantt-type timeline was created using Python (v3.11) and Matplotlib (v3.8) to illustrate the temporal distribution and potential overlap of CRKP and VRE isolations in newborn patients. The timeline for each patient was considered as the date of birth and the start of hospitalization.

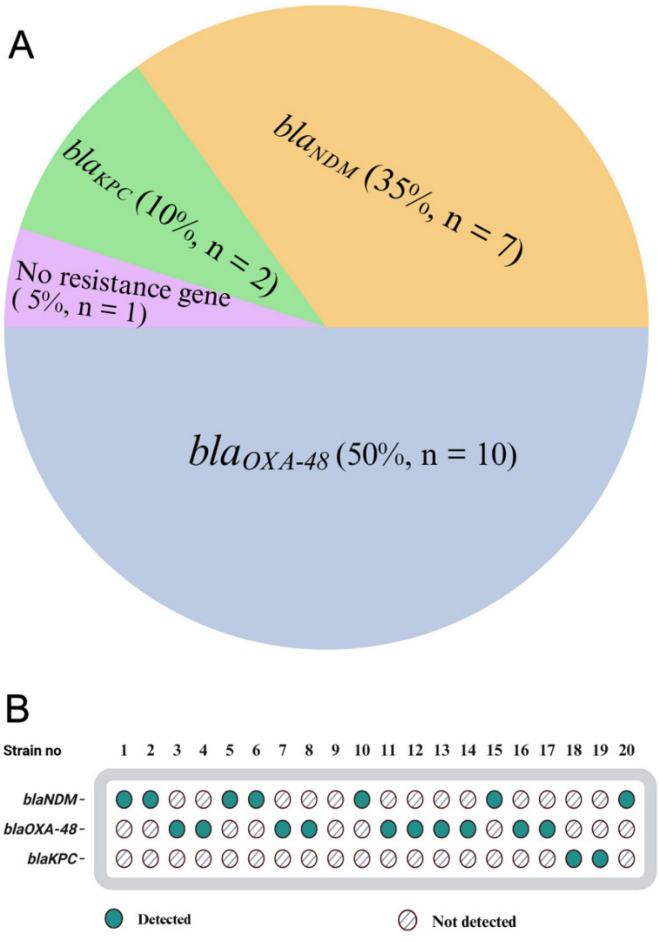
Results

Antibiotic Susceptibility Testing Results

A total of 20 preterms were evaluated in our study. All VRE strains included in the study were resistant to both glycopeptides (vancomycin and teicoplanin). There was no growth inhibition zone around vancomycin and teicoplanin disks for VRE isolates. Based on their isolation dates, it was determined that 17 preterms were colonized with VRE or CRKP at least once. In 9 of these infants, the first isolated strain was

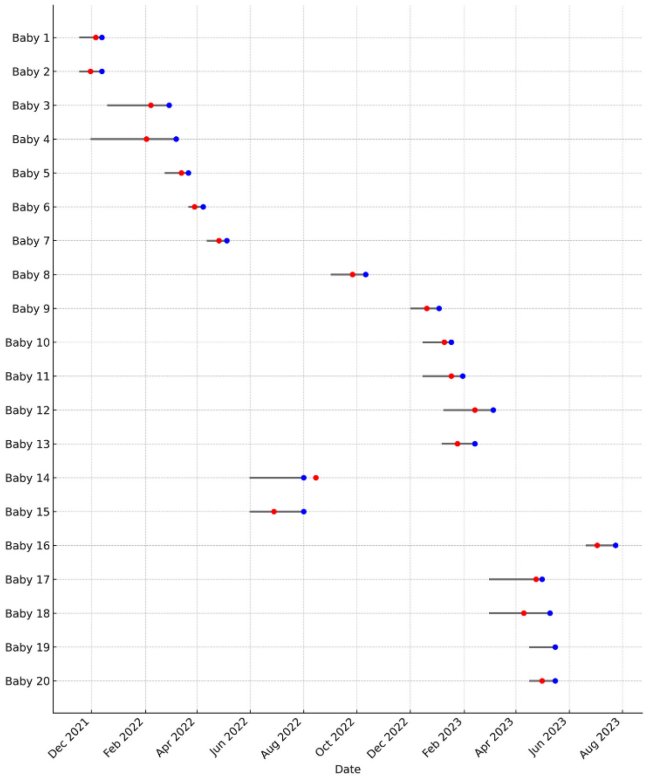
CRKP, in 7 VRE, and in 1, both pathogens were isolated simultaneously. In 6 infants (30%), both pathogens (VRE and CRKP) were isolated together. In these patients, double colonization mostly developed within 1 to 3 weeks after the isolation of the first pathogen. Sequential colonization was particularly striking in infants 2, 11, and 18. The mean time between the date of birth and the first isolation date was calculated as 18.2 days for CRKP and 24.5 days for VRE. In the only infant (Baby 20) in whom both pathogens were isolated simultaneously, isolation occurred 29 days after birth. A detailed examination of the data reveals that in some infants, two different isolates were identified at different times. For example, in infants 2 and 11, CRKP was detected first, and then VRE. In infants 15 and 18, VRE was isolated on two separate dates (Figure 2).

Figure 3A. Distribution of carbapenemase genes among *K. pneumoniae* isolates; **B.** Carbapenemase genes detected in strains.



No carbapenemase resistance gene was detected in baby number 9.

Figure 2. Gantt chart showing the temporal distribution of CRKP and VRE isolations in preterm infants included in the study.



Each horizontal line represents the time between the patient's date of birth (start of hospitalization) and the date of first isolation. Dots are the isolation dates: blue (VRE), red (CRKP).

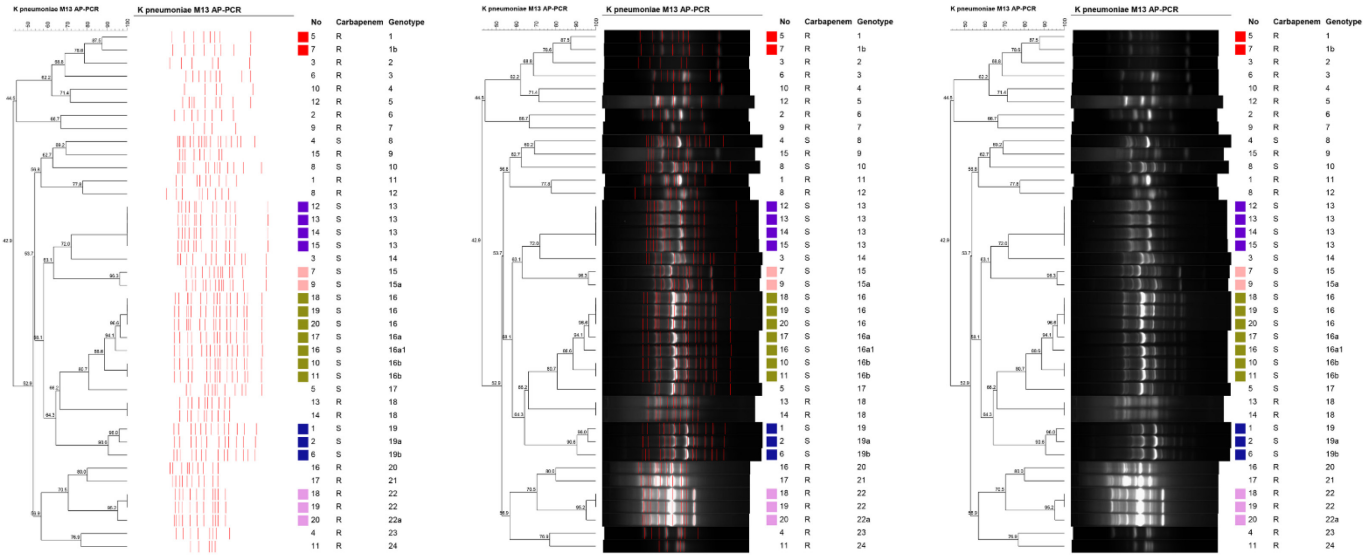
Antibiotic Resistance Genes Detection and PFGE Results

No genetic relationship was detected between 20 CSKP, 20 VSE (meconium isolates), and 20 CRKP, 20 VRE (fecal isolates), respectively, which revealed that carbapenem resistance in *K. pneumoiae* and vancomycin resistance in *Enterococcus* strains were not developed as a result of antibiotic treatment, i.e., the source of CRKP and VRE was exogenous, not endogenous. Detection of different genes carried on plasmids, such as major carbapenemase genes in CRKP and *van* genes in VRE strains, showed the exogenous source of colonization of both multidrug pathogens in the intestine of preterm babies. We detected the type of gene encoded carbapenemase in 19 out of 20 CRKP. bla_{OXA-48} gene was detected in 10 (53%), bla_{NDM} in seven (37%), and bla_{KPC} in two babies (% 10%) (Figure 3). Also, *vanA* was the predominant gene in 17 out of 20 VRE strains. Throughout the study period, 40 *K. pneumoniae* isolates were analyzed, and no predominant outbreak strain was identified. Among the 40 isolates, 24 distinct genotypes were detected. The

isolates showing clustering were grouped into seven distinct clusters. Of the isolates, 23 were part of a cluster, resulting in a clustering rate of 57.5%. The largest cluster was Genotype 16, comprising seven isolates.

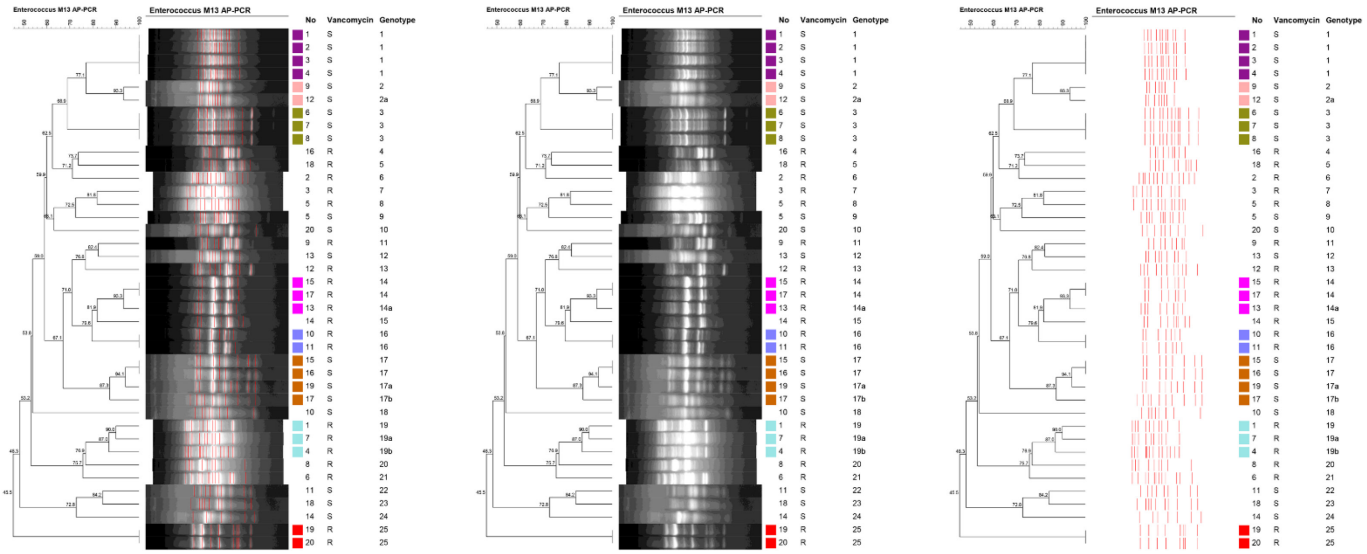
Carbapenem-resistant strains were divided into 16 distinct genotypes, and susceptible strains were divided into 8 different genotypes. bla_{NDM}-type carbapenem resistance gene was detected in 7 CRKP strains, bla_{OXA-48}-type in 10, and bla_{KPC}-type in 2. No resistance gene was detected in the CRKP strain isolated from baby no 9. The same type of resistance gene (bla_{NDM}) was detected in babies 1 and 2 of the twins, and different types of resistance genes were detected in all other twins. Among the carbapenem-susceptible strains, 4 infants (baby no. 12, 13, 14, 15) were identified as genotype 13, and 7 infants (baby no. 10, 11, 16, 17, 18, 19, 20) were identified as genotype 16 (Figure 4).

Figure 4. The dendrogram is derived from PFGE pattern analysis of CRKP strains.



The following data is presented: the distribution of PFGE types among CRKP strains, the resistance genes detected, and the isolation date. The letters "a" and "b" represent strains that show a very close kinship but are not identical.

Figure 5. The dendrogram is derived from PFGE pattern analysis of VRE strains.



The following data is presented: the distribution of PFGE types among VRE strains, the resistance genes detected, and the isolation date. The letters "a" and "b" represent strains that show a very close kinship but are not identical.

Similarly, in the study involving 40 *Enterococcus* isolates, no predominant outbreak strain was identified. Among these isolates, 25 distinct genotypes were detected. Isolates showing clustering were grouped into eight distinct clusters. Of the isolates, 23 were part of a cluster, yielding a clustering rate of 57.5%. The largest clusters were Genotype 1 and Genotype 17, each containing four isolates.

All strains within the clusters were considered closely related. Our findings demonstrate the presence of polyclonal diversity among the isolates. The *vanA*-type resistance gene was detected in all strains resistant to vancomycin. *vanA, vanB*, and v*anC* genes were not detected in only 3 VRE strains. The highest grouping among VRE strains was detected in genotype 14 (baby no. 13, 15, 17) and genotype 19 (baby no. 1, 4, 7) (Figure 5).

Discussion

Preterm infants are admitted to intensive care units because many systems have not yet fully developed, and their developmental processes are meticulously monitored [23]. Intensive care units are areas where drug-resistant bacteria can easily spread between individuals and cause outbreaks. Since the intestinal microbiota in preterm babies is not fully developed, these bacteria can easily colonize and, if not isolated, can spread to other babies in the unit via healthcare workers [24-26]. The continuous rise in VRE since they were first described in the mid-1980s and the alarming increase in carbapenem-resistant *Enterobacteriaceae* (CRE), especially CRKP, in recent years, are considered serious public health threats and growing problems in hospitals worldwide [26,27]. As they become increasingly resistant to current treatment drugs, the treatment of infections caused by these MDR organisms is very challenging [28-30]. Studies have shown that these infections are responsible for 35% of newborn deaths [31,32]. For this reason, these two opportunistic infectious bacteria have been added to the priority pathogen list by the World Health Organization [33]. It is important to know the source of colonization of CRKP and VRE. If colonization is endogenous, revision of antibiotic protocols administered to preterm infants during intensive care hospitalization should be considered to delay the development of carbapenem resistance in *Klebsiella pneumoniae* and vancomycin resistance in *Enterococcus*. Otherwise, in exogenous sources, handwashing of healthcare workers is enough to treat such colonization. In this study, 20 carbapenem-susceptible and vancomycin-susceptible meconium isolates were analyzed and compared with 20 CRKP and VRE isolates obtained from the same infants. The AP-PCR method was used to examine the clonality between isolates, besides the gold standard method, the PFGE. AP-PCR stands out as a fast and cost-effective alternative to PFGE, especially in differentiating genetically independent isolates. Numerous studies in the literature demonstrate that both methods provide consistent results. The efficiency of AP-PCR in terms of time and cost, its technical ease, and its successful application under properly optimized laboratory conditions were key factors in our decision to choose this method. The findings indicate that CRKP and VRE colonization originated from exogenous sources, most likely related to inadequate hand hygiene practices. Seventeen infants were colonized with CRKP, and VRE isolates harboring carbapenemase-encoding *van* genes. The bla_{OXA-48} was the predominant gene in 10 infants, followed by seven bla_{NDM}, and two $bla_{KPC,}$ opposite to our previous study in infants reported by Demir *et al.* in 2023 [34], which revealed bla_{NDM} (42%) as the most dominant, followed by bla_{OXA-48} in 16%, and bla_{VIM} in one (2%) isolate, and bla_{NDM} + bla_{OXA-48} co-existed in 36% of the *Klebsiella* isolates [35]. The composition of the gut microbiota is vulnerable to many factors, such as antibiotic administration and other clinical treatments [36]. A previous study showed that giving antibiotics to mice made it possible for VRE to quickly and completely replace the normal gut microbiota [37]. Also, anti-anaerobic antibiotic treatment disrupted the stability of the gut microbiota by significantly reducing the abundance of anaerobic bacteria, leading to CRKP colonization [38]. A study conducted in Japan utilized PFGE on 6,000 rectal samples and identified 60 VRE, which were categorized into three primary clusters and four multistrain clusters (the first main cluster comprised 26 isolates, the second contained 10 isolates, the third consisted of 6 isolates, and the remaining 4 clusters comprised 2 isolates each). In accordance with the PFGE pattern, this case was interpreted as a nosocomial infection, according to the presence of multiple clones [39]. The study investigating the genetic affiliation of VRE strains using PFGE and multi-locus sequence typing (MLST) methods in more than a thousand samples collected from 3 different hospitals in Iran showed that there was intra-hospital and inter-hospital circulation of VRE and similar pulsotypes and STs among different sources [40]. Additionally, a study carried out in 2014 using VRE strains isolated from rectal swab samples collected from babies hospitalized in the neonatal intensive care unit in Türkiye determined that the strains had two main origins [41].

In this study, among 20 VRE isolates, 17 were carrying the *vanA* gene on plasmids, as expected based on resistance to both vancomycin and teicoplanin. *vanA-B-C* type resistance genes were not detected in three isolates, but resistance to vancomycin was detected. This resistance may be caused by genes causing vancomycin resistance other than *vanA-B-C* genes, such as *vanH* and *vanX*. Among twin babies, VRE of the same origin as the *vanA* gene was detected in babies numbered 10-11. Although VRE of the same origin was detected in babies numbered 19-20, none of the *vanA-B-C* genes were detected. All VRE isolates detected in the other two infants were identified as having different origins.

Despite the fact that carbapenems represent the most extensively utilized class of antimicrobial agents among the group of antibacterial drugs designed for the treatment of *Enterobacteriaceae* clinical isolates, cases of infections caused by CRE are increasing on an annual basis, with the associated morbidity and mortality rates being significant [42]. Among the carbapenem resistance genes, $bla_{KPC,}$ $bla_{OXA-48,}$ and bla_{NDM} are the most common genes in the world [43,44]. Fisher *et al.* [45] stated that bla_{OXA-48} and bla_{NDM} were the two main carbapenemase genes detected in Malaysia. Previous studies have shown that the resistance mechanism of carbapenem-resistant *Enterobacteriaceae* clinical strains isolated from pediatric patients is different. In China, for CRE strains isolated from pediatric patients, the main type of carbapenemases mediating resistance to carbapenems was metallo-β-lactamases, including bla_{NDM} and bla_{IMP} [46]. In the outbreak analysis conducted in Japan by Yamagishi *et al.*, it was reported that the bla_{IMP}-type carbapenemase gene was detected in all of the strains included in the study (n = 22) [47]. In the study conducted by McKenney *et al.* [48], carbapenemase genes were detected in 55 isolates, with the dominant carbapenemase gene being bla_{OXA-48} (63.5%), followed by bla_{NDM} (36.5%). A total of 63 isolates were grouped into 30 clusters, exhibiting 80% similarity as determined by PFGE analysis. In the study conducted by Miao *et al.* [2] in China, various carbapenemases were identified, including bla_{KPC} (n = 46), bla_{NDM} (n = 7), bla_{IMP} (n = 2), and bla_{VIM} (n = 2) were identified among 54 CPE isolates from patients treated in a district hospital. No strains carrying bla_{OXA-48}-like genes were found. Among the identified CPE, bla_{KPC} was the most prevalent, predominantly present in CRKP.

This study was conducted in a resource-limited setting, where access to whole-genome sequencing was not available at the time of investigation. PFGE was selected under the national surveillance protocols and was the most discriminative and standardized typing method available to us during the study period. PFGE remains a valid and widely referenced typing method in many surveillance networks, including those coordinated by the CDC and ECDC, particularly in retrospective studies or when whole genome sequencing (WGS) is not accessible.

This study has several limitations. First, the relatively small sample size (n = 20) and single-center design may limit the generalizability of the findings. Second, clinical outcome data such as infection progression, morbidity, or mortality were not integrated, reducing clinical relevance. Third, the absence of WGS restricted detailed analysis of transmission dynamics. Future multicenter studies incorporating larger cohorts, clinical outcomes, and advanced molecular techniques are recommended. Additionally, the lack of clinical outcome data, such as progression to infection, morbidity, or mortality, limits the translational impact of the findings. Incorporating WGS or other advanced molecular techniques would enhance understanding of resistance mechanisms and transmission dynamics. Future studies should be multicentric, involve larger patient populations, and integrate longitudinal clinical follow-up to assess outcomes related to CRKP and VRE colonization.

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Ethical Approval

This study followed the Declaration of Helsinki and was approved by the Ethics Committee of Istanbul Medical Faculty (approval number E-29624016-050.99-1081314).

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Conflict of interest

No conflict of interest is declared.

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